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Research Article

Physicochemical Characterization of Altebrel™, a Proposed Etanercept Biosimilar

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Background: Etanercept is prescribed for the rapid and effective treatment of chronic immune-mediated inflammatory disorders. Due to the expiration of etanercept patents and worldwide demand for comparable and more affordable substitutes, several biosimilars of etanercept have been approved in different countries and new ones are in the process of approval.
Objectives: In the present study, Altebrel™ as an etanercept proposed biosimilar was investigated in a side by side comparison using various orthogonal analytical methods.
Materials and Methods: Three batches of the Altebrel™ and Enbrel® samples were used for the study. Several physicochemical properties of samples were compared according to international guidelines, incliding; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Capillary electrophoresis sodium dodecyl sulfate (CE-SDS), size exclusion high performance liquid chromatography (SE-HPLC), hydrophobic interaction chromatography high performance liquid chromatography (HIC-HPLC) and its biological activity was evaluated using surface plasmon resonance affinity analysis and tumor necrosis factor alpha (TNFα) neutralization biological assay. Amino acid analysis was applied to check the primary sequence and far-UV circular dichroism (CD) spectroscopy investigated the secondary structure.
Results: The obtained results indicated a high degree of similarity between Altebrel™ and Enbrel®. Results of SDS-PAGE, CE-SDS, HIC-HPLC and SE-HPLC implied a comparable pattern of size variants for all samples. Based on the data achieved via *in vitro* bioactivity assays and SPR analysis, the functional property of Altebrel™ was proved comparable to that of the reference product. Moreover, amino acid analysis indicated similar primary structure and circular dichroism study implied a similar secondary structure for Altebrel™ and Enbrel®.
Conclusion: Overall, our data provide analytical evidence for structural and *in vitro* functional similarity between Altebrel™ and Enbrel®.
Keywords: Etanercept, Biosimilar, Comparability study, Physicochemical Characterization

1. Background

Etanercept (Enbrel®, Amgen-Pfizer) has received a great deal of attention as one of the first USA food and drug organization (FDA)-approved tumor necrosis factor alpha (TNFα) antagonist for the treatment of rheumatoid arthritis (1). This recombinant dimeric human TNFα receptor is fused to the Fc portion of human IgG1, which inhibits TNFα-induced inflammation (2). It is licensed to be prescribed in rheumatoid arthritis, certain forms of juvenile idiopathic arthritis, ankylosing spondylitis, psoriatic arthritis, Crohn's disease, plaque psoriasis, and inflammatory bowel disease, which TNFα is overexpressed in all of them (3,4). The successful worldwide sale of etanercept has led to the efforts on the advent of many biosimilars by various biopharmaceutical companies in around the world (5).

A recombinant medicinal molecule with similar quality, efficacy and safety compared with a commercialized reference biopharmaceutical, is referred as biosimilar (6). It is a biological product that is highly similar to and has no clinically meaningful differences from an existing reference product (7,8). In order to demonstrate the biosimilarity of a biomolecule in relation to the original one, a series of *in vitro* and *in vivo* studies including quality testing, pre-clinical and clinical trials, toxicology, tolerability, pharmacokinetics, and pharmacodynamics are instructed by the regulatory authorities (9–11). The guidelines on evaluation of biosimilarity vary in different countries but usually revolve around the principle of lacking clinically meaningful differences in terms of safety, efficacy, and quality (11). Expiration of the issued patents for some biopharmaceuticals offers a

golden opportunity for the development of biosimilars to exploit a previously established market (5). The raised economic competition between a reference medicine and its biosimilars will benefit the healthcare system through alleviation of medical expenses associated with the drug and facilitates patient access to more affordable prescriptions (12).

2. Objectives

For having a better view on the etanercept structural and functional properties, and with an understanding of which quality features are clinically relevant, a comparability study was performed on Altebrel™ (etanercept proposed biosimilar) and Enbrel®. Therefore, the quality attributes of Altebrel™ and Enbrel® were compared using various orthogonal methods. The purity and structural properties were assayed by application of SDS-PAGE, Capillary electrophoresis sodium dodecyl sulfate (CE-SDS), hydrophobic interaction chromatography high performance liquid chromatography (HIC-HPLC), size exclusion high performance liquid chromatography (SE-HPLC), circular dichroism (CD) and amino acid analysis. Functional assays such as SPR affinity analysis and biological assay were also conducted for assessing the biological activity of Altebrel™ in comparison with Enbrel®. Biosimilarity of Altebrel™ and the branded drug was evaluated considering guidelines and publications provided by the innovator company (13) and those issued by European medicines agency (EMA) (6) and FDA (7) for the production of biosimilars. Our findings indicated a high similarity between Altebrel™ and Enbrel®.

3. Materials and Methods

3.1. Reagents and Materials

Three different lots of Enbrel® (G30909, H17609, H42831; Pfizer, USA) and Altebrel™ (9202006, 9202008, 9202009; Aryogen Pharmed, Iran) were used in all the experiments. TNFα, Alamar blue dye, and human antibody capture kit were purchased from Merck Millipore (Germany), Invitrogen (now a part of GE Healthcare) and GE Healthcare Life Sciences (USA), respectively. Organic solvents, acrylamide, bisacrylamide, tris sodium salt, urea, Dithiothreitol, Tetramethylethylenediamine, ammonium persulphate, iodoacetamide, bovine trypsin, ampholytes, β-1,4-galactosidase, b1-R-N-acetylglucosaminidase, and α2-3,6,8,9-neuraminidase were of the HPLC grade.

3.2. Determination of Concentration

The concentration of etanercept samples was quantified by UV-vis spectroscopy using a PerkinElmer Lambda25 UV-Vis spectrophotometer (PerkinElmer, USA). The samples were measured in 2-mL half-micro quartz cuvettes (PerkinElmer, USA) with a path length of 10 mm. The absorbance of samples at 280 nm was measured. Background correction was performed with formulation buffer without any protein. The extinction coefficient (ε) was considered 1.14. The samples were analyzed in triplicates.

3.3. SDS-PAGE

Protein samples (5 µg) were prepared by diluting in 2x sample buffer in the non-reduced condition. In the reduced condition, they were heated at 95 °C for 5 min in the presence of 2-mercaptoethanol. The samples were separated in 10% SDS gel at constant 100V. The gels were stained using Coomassie blue solution and were scanned after destaining, and analyzed by Bio-Rad Quantity One 1-D software (Bio-Rad, USA).

3.4. Capillary Gel Electrophoresis (CE-SDS)

Capillary gel electrophoresis was conducted with Agilent 7000 CE instrument equipped with a photodiode array (PDA) detector. The samples were analyzed based on Beckman Coulter application note (14).

3.5. Size Exclusion Chromatography

The profile of lower and higher molecular weight components of the drug samples were measured by Knauer HPLC instrument (Knauer, Germany) using a TSKgel G3000 SWXL (Tosoh, Japan) analytical column (5 µ, 7.8×300 mm) and a flow rate of 1.0 $mL.min^{-1}$ over 50 min, with a mobile phase of 0.2 M phosphate buffer pH 7.1 at 25 °C. Twenty µg of samples were injected and UV absorption was detected at 280 nm. Results have been expressed as relative area under the curves of different samples. The samples were injected and analyzed in triplicate.

3.6. Hydrophobic Interaction Chromatography

Knauer HPLC instrument was applied for HIC-HPLC analysis of the samples. The proteins were diluted with distilled water to a concentration of 1 $mg.mL^{-1}$ and 10 µg was injected onto an analytical HPLC column (TSK Gel Butyl-NPR, 4.6 mm ID × 100 mm) at 30 °C. Product-related impurities were separated in a linear gradient using mobile phase A composed of 1.8 M ammonium sulfate pH 7 with sodium phosphate 0.1 M, pH 7 and mobile phase B composed of disodium hydrogen phosphate (0.1 M, pH 7). The flow rate was 1.0 $mL.min^{-1}$ for 50 min, and the chromatography was monitored by fluorescence detector (excitation of 278 nm and emission of 350 nm). The samples were assayed in triplicate.

3.7. Surface Plasmon Resonance

The affinity and kinetics of the ligand interaction with Altebrel™ and Enbrel® samples were studied by application of surface plasmon resonance using the capturing method (Human antibody capture kit) by GE Healthcare BiacoreTM X100 (GE Healthcare) in multi-cycle kinetics analysis. First, the anti-human IgG1 antibody was immobilized in an amount of 800 RU on the CM5 chip. Then, etanercept was captured by immobilized anti-IgG1 antibody in amount of 150 to 170 RU. Finally, 2-fold serially diluted TNFα from 0.125 nM to 8 nM concentrations were sequentially injected at a constant flow rate into the flow cell for 180 s in the association phase and 2400 s in the dissociation phase to perform surface-bound analysis for obtaining the multi-cycle association and dissociation sensorgrams. HBS-EP buffer (10 mM HEPES, pH 7.4; 150 mM NaCl; 3 mM EDTA; 0.005% Tween-20) was used for diluting the samples and as the running buffer. For regeneration of the sensor chip surface, 5 M $MgCl_2$ for 60 s was injected at the end of each cycle. All the analyses were carried out in triplicate. The binding kinetic parameters including Ka (association rate constant), Kd (dissociation rate constant) and KD (dissociation equilibrium constant) were fitted to the 1:1 Langmuir interaction model calculated by BIAevaluation software version 2 (GE Healthcare).

3.8. Biological Assay

Neutralization of TNFα by etanercept results in the prevention of L929 murine fibroblast necroptosis. This was the basis for setting up the biological assay analysis. 50 µL of TNFα (25 $ng.mL^{-1}$) was transferred into a 96-well microplate containing 8×10^4 of L929 cells per well in DMEM-F12 medium supplemented with 10% FBS. Then, 100 mL of serially diluted Altebrel™ and Enbrel® samples was added to each well and incubated for 72 h at 37 °C, 5 % CO2 and 95-98 % humidity condition. After incubation, 30 µL of Alamar blue dye (cell viability indicator) were added and incubated for eight hours. The fluorescence was measured with a BioTek FLx800 fluorescence microplate reader (USA) at 540 nm for excitation and 590 nm for emission.

3.9. Circular Dichroism

Far-UV circular dichroism spectroscopy was applied for evaluation of the higher order structure of etanercept. Jasco J-810 spectropolarimeter (Tokyo, Japan) was used for getting the CD spectra. Concentrations of the samples were set up to 0.2 $mg.mL^{-1}$. By using a step size of 1 nm and a bandwidth of 1.5 nm, the far-UV spectra were recorded in a cell with a path length of 1 mm at 22 °C. The spectra were corrected for buffer contributions and the CD-spectra were analyzed using the program Spectra Manager for Windows 95/NT, spectra analysis version 1.53.02, JASCO Corporation.

3.10. Amino Acid Analysis

For quantification and comparison of amino acid content of the samples, after acid hydrolysis, o-Phthalaldehyde derivatization was chosen. After derivatization, they were separated by reversed phase high performance liquid chromatography (RP-HPLC) (Knauer, Germany) and detected by a fluorescence detector. The whole procedure was based on Bidlingmeyer *et al*. described method (15).

4. Results

4.1. Concentration Determination

Among many routine measurement methods, UV-vis spectroscopy presents a relatively accurate and reliable approach to quantify the concentration of biologics in a solution. The obtained spectroscopy results were similar for the reference and biosimilar samples and all Altebrel™ batches showed the similar concentration in the range of 45 to 55 $mg.mL^{-1}$.

4.2. Size Variants Analysis

Figure 1 shows the SDS-PAGE analysis of the reference and proposed biosimilar samples. In **Figure 1**, lanes 1 and 4 are the reduced Enbrel G30909 and Altebrel 9202009 respectively. Lanes 2 and 5 are 6% of the total protein which was run in lanes 1 and 4 respectively. It is the same for lanes 3 and 6, except they contain 8% of the total protein run in lanes 1 and 4. By relative comparison of the total intensities of the high molecular weight component (HMWC) and low molecular weight component (LMWC) in lanes 1 and 4 with lanes 2 and 5 or 3 and 6, it can be assured that the HMWC or LMWC are less than 6% or 8% of the total protein (In lanes 1 and 4) respectively. Densitometry of the HMWC and LMWC impurities can be seen in the relative percentage of the mentioned band to the sum of the whole intensities of the all bands in lanes 1 and 4. The results can be found in **Table 1**.

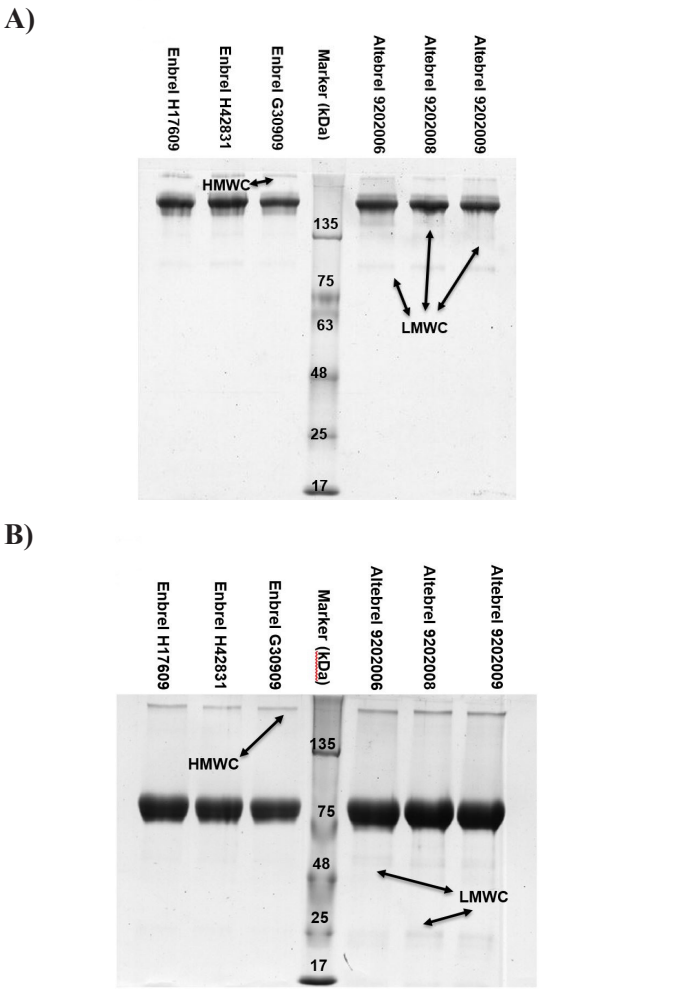


Figure 1. A) Non-reduced and **B)** Reduced samples of Enbrel® and Altebrel™. **C)** SDS-PAGE densitometry of Enbrel G30909 and Altebrel 9202009 in percentage. Lanes 1 to 3 are Enbrel G30909, 6% (0.3 µg) and 8% (0.4 µg) of Enbrel G30909 respectively. Lanes 4 to 6 are Altebrel 9202009 (5 µg), 6% (0.3 µg) and 8% (0.4 µg) of Altebrel 9202009 respectively.

Capillary electrophoresis was also performed to evaluate the electrophoretic profiles of the etanercept samples. **Figure 2** illustrates the migration profile of the samples.

In order to further analysis and separate the lower and higher molecular weight components in the branded and biosimilar product, SE-HPLC was performed. The gained chromatograms of Altebrel™ and Enbrel® test samples were determined by comparing the AUC of the major peaks together. **Figure 3** and **Table 2** show the results.

Table 1. SDS-PAGE densitometry of Altebrel™ and Enbrel®. The main band and the related impurities show the ratio of each band to the whole bands in percentage.

Sample	HMWC %	LMWC %	Main Band
Altebrel 9202006	3	7.1	89.9
Altebrel 9202008	3.3	8.5	88.2
Altebrel 9202009	4.1	6.4	89.5
Enbrel G30909	4.7	7.4	87.9
Enbrel H17609	3.5	6.5	90
Enbrel H42831	2.1	9.9	88

Table 2. Relative percentage of the peak A + A' based on AUC of the peaks as an indicator of purity in Enbrel® and Altebrel™ samples using SE-HPLC analysis.

Sample	Altebrel 9202006	Altebrel 9202008	Altebrel 9202009	Enbrel G30909	Enbrel H17609	Enbrel H42831
Peak A + A' (%)	95.87 ± 0.08	97.07 ± 0.03	96.37 ± 0.30	96.28 ± 0.07	96.68 ± 0.05	96.25 ± 0.06

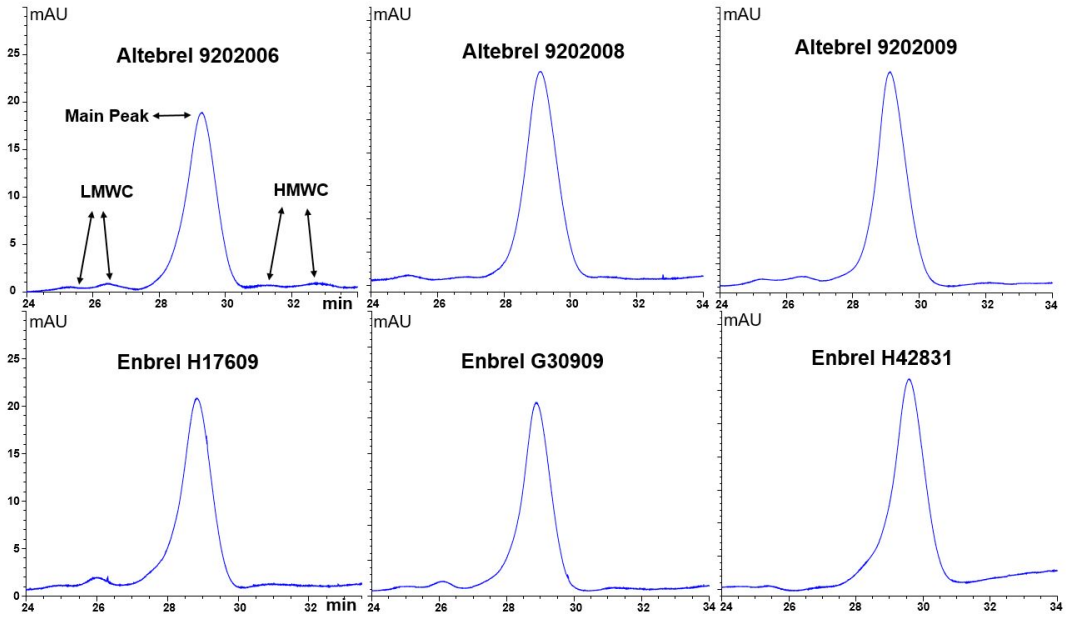


Figure 2. Non-reduced capillary gel electrophoresis analysis of etanercept samples. Etanercept electropherograms show the HMWC, main peak and LMWC.

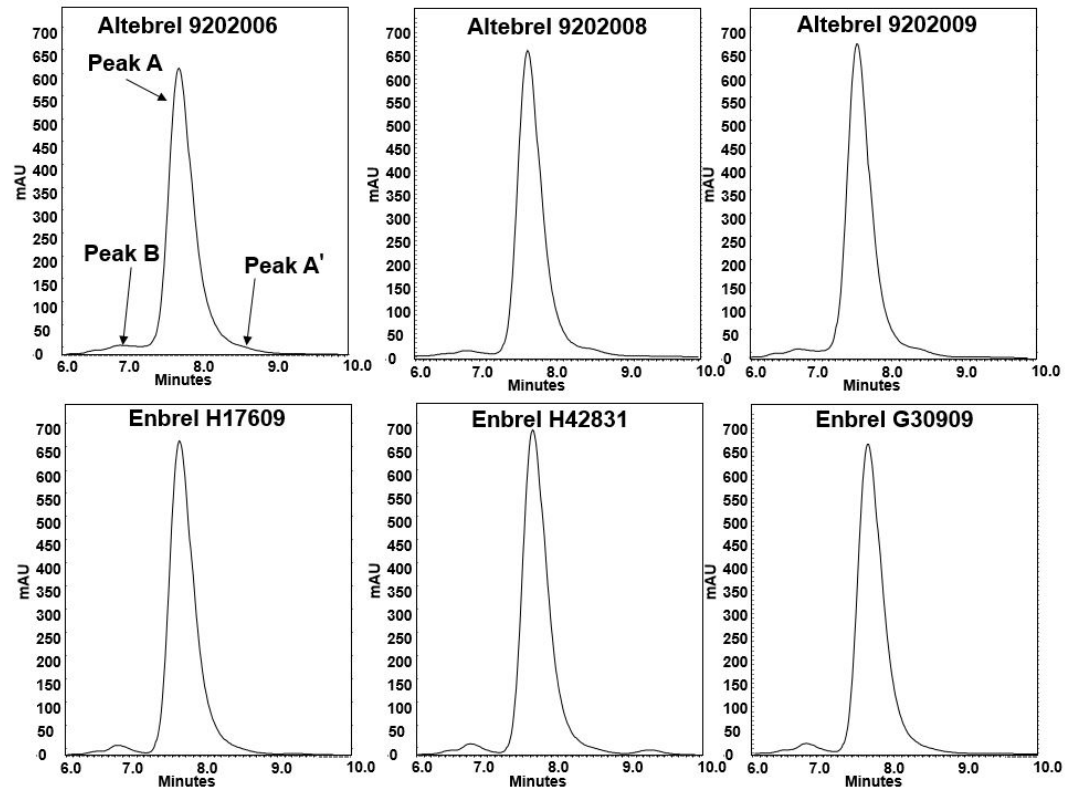


Figure 3. SE-HPLC analysis of Altebrel™ and Enbrel® samples. The samples were separated into three major areas; Peak A is the main sample, Peak A' is the degraded form and Peak B is the aggregated form.

In the next step, HIC-HPLC was performed to have more data about purity of etanercept samples. Figure 4 displays the HIC-HPLC chromatograms of Altebrel™ and Enbrel®. HIC-HPLC separates etanercept into peak 1 (mainly cleaved species), peak 2 (main product), and peak 3 (misfolded species and aggregates) (16). As truncated forms are less hydrophobic, they appear first in the chromatogram and are followed by the main peak, and misfolded forms, which are more hydrophobic.

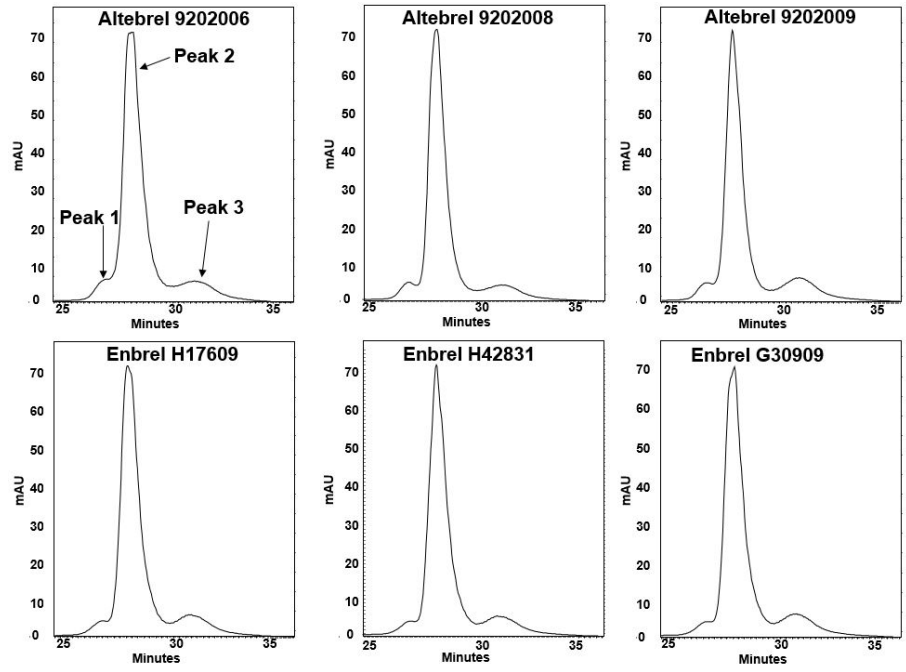


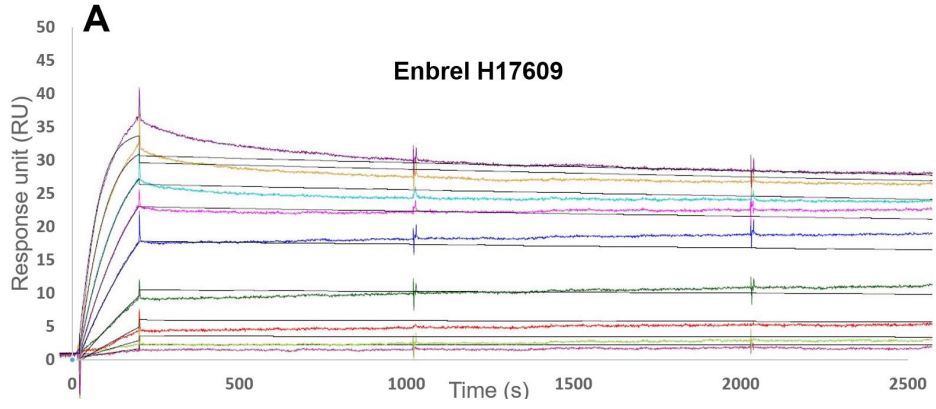
Figure 4. HIC-HPLC analysis of Altebrel™ and Enbrel® samples. Peak 1: degraded and truncated species; Peak 2: Main product; and Peak 3: misfolded and aggregated species.

4.3. In-Vitro Functional Analysis

Functional analysis of etanercept was performed using two analytical methods. SPR was applied to study the kinetics of etanercept binding to TNFα and a bioassay was set up to evaluate the power of the samples to neutralize the necroptotic effect of TNFα on L929 cells. As it can be inferred from figure 5, the increasing concentrations of the flowed TNFα from 0.125 nM to 8 nM over the captured etanercept, leads to the saturation of etanercept molecules with TNFα on the chip surface. In addition, the dissociation time was set up to 1800 s to allow the instrument for gathering the enough data for measuring the dissociation rate constants. **Table 3** shows the binding affinity of Enbrel® and Altebrel™ samples to TNFα.

Table 3. The measured KD values of Enbrel® and Altebrel™ binding affinity to TNFα, based on SPR analysis.

Samples	Altebrel (9202009)	Altebrel (9202008)	Altebrel (9202006)	Enbrel (G30909)	Enbrel (H42831)	Enbrel (H17609)
KD	(3.21 ± 0.12) E-11	(3.39 ± 0.10) E-11	(2.89 ± 0.09) E-11	(2.77 ± 0.09) E-11	(2.66 ± 0.03) E-11	(1.80 ± 0.25) E-11



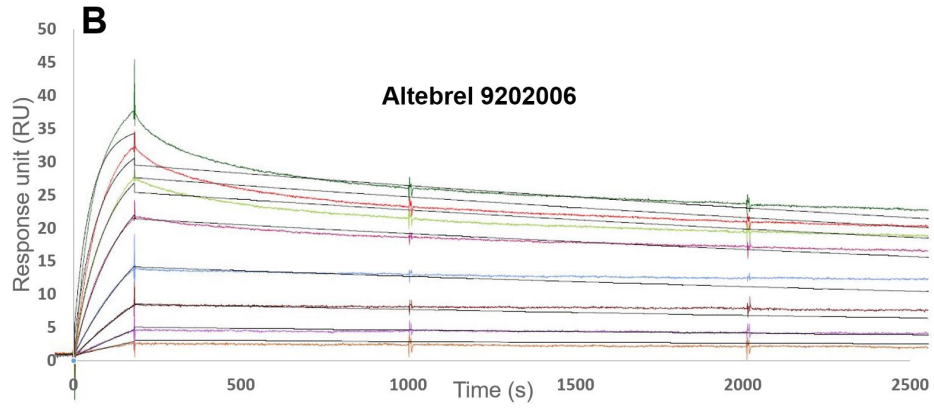


Figure 5. Sensorgrams from SPR analysis of A) Enbrel® and B) Altebrel™ as an example. Appropriate curvature of the sensorgram in the association and dissociation time would result in gaining the reliable results.

The potency of Enbrel® and Altebrel™ to neutralize the necroptotic effect of TNFα on L929 mouse fibroblasts was measured through the increasing concentrations of etanercept which should elevate the cell survival rate in the presence of TNFα. After treatment of L929 cells with serially diluted Altebrel™ and Enbrel® at the constant concentration of TNFα, the rates of cell survival for all six drug samples were assayed as shown in **Figure 6** and **Table 4**.

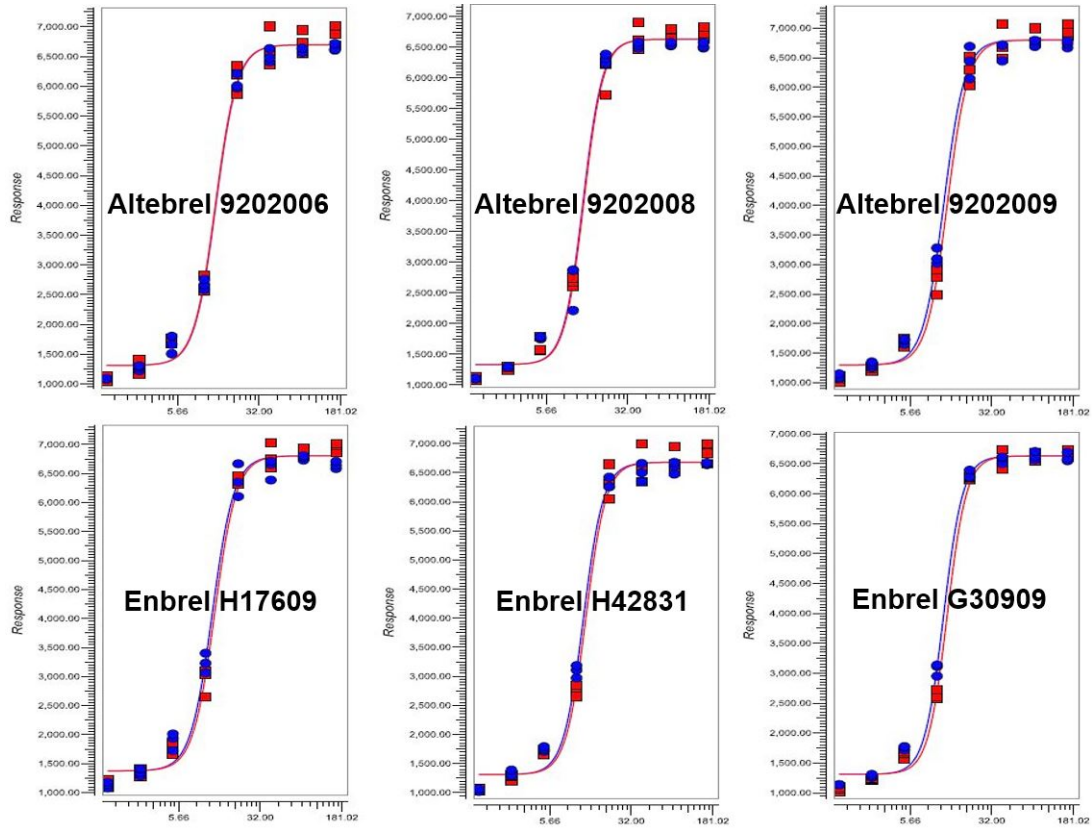


Figure 6. Biological assay of etanercept samples. In all of the samples, reference standard is pool of the three Enbrel® samples.

Table 4. Potency ratios of Enbrel® and Altebrel™ samples based on biological assay.

Samples	Altebrel (9202009)	Altebrel (9202008)	Altebrel (9202006)	Enbrel (G30909)	Enbrel (H42831)	Enbrel (H17609)
Potency ratio	0.975 ± 0.10	1.037 ± 0.022	1.012 ± 0.20	0.995 ± 0.56	1.059 ± 0.20	1.018 ± 0.34

4.4. Secondary Structural Analysis

Herein, the secondary structure was determined using far-UV CD. This method quantifies the differences in left and right-handed circularly polarized light in a spectrum in the far-UV range (190 - 250 nm). Results from far-UV CD spectroscopy are provided in **Table 5**.

Table 5. Secondary structure of Altebrel™ and Enbrel® samples, measured by far-UV CD spectroscopy.

Samples	Altebrel 9202006	Altebrel 9202008	Altebrel 9202009	Enbrel H17609	Enbrel H42831	Enbrel G30909
A (%)	9.8 ± 0.5	9.2 ± 0.7	8.7 ± 2	7.0 ± 0.8	8.7 ± 1	7.9 ± 1.1
B (%)	23.1 ± 3.8	31.1 ± 5.2	32 ± 4.8	38.3 ± 5.5	31.5 ± 2.7	31.1 ± 3.9
Turn (%)	24.7 ± 0.4	21.4 ± 3.9	17.5 ± 1	17.3 ± 2.7	21.5 ± 3.4	19.9 ± 1.3
Random (%)	42.3 ± 2.5	38.1 ± 2.1	41.8 ± 4.6	37.2 ± 2.5	38.2 ± 0.9	41.2 ± 0.4

4.5. Amino Acid Analysis

Amino acid analysis is used to evaluation of the amino acid content of the proteins. In this method, a protein is hydrolyzed to its individual amino acid constituents. After that, the amino acids will be derivatized for RP-HPLC analysis. After setting up the acid hydrolysis time and the sample concentration, 15 amino acids were quantified and compared in Altebrel™ and Enbrel® samples. The results were gathered in **table 6**.

Table 6. Amino acid analysis of Altebrel™ and Enbrel® by RP-HPLC. A1, A2 and A3 are the Altebrel 9202006, Altebrel 9202008 and Altebrel 9202009 samples respectively. E1, E2 and E3 are the Enbrel G30909, Enbrel H17609 and Enbrel H42831 respectively. The known and experimental values of each amino acid are shown. Moreover, average of the compositional error of Altebrel™ and Enbrel® samples was calculated and shown in percentage separately.

Amino Acid	Known Value	Experimental Value (average)						Average of Comp. error of A1, A2 and A3 (%)	Average of Comp. error of E1, E2 and E3 (%)
		A1	A2	A3	E1	E2	E3		
D+N	70	72.00	75.17	75.50	77.68	74.82	69.35	6.033	5.64
E+Q	98	108.8	111.4	111.5	109.7	100.5	103.1	12.823	6.56
S	94	92.42	92.41	92.86	95.99	94.81	96.36	-1.528	1.83
H	22	21.0	20.6	20.7	21.5	20.8	21.5	-5.606	-3.33
G	48	42.2	44.3	38.0	44.8	46.9	48.0	-13.542	-2.99
T	84	75.9	75.4	76.1	78.5	78.4	81.2	-9.762	-5.52
R	38	91.9	90.2	91.3	39.3	39.3	40.4	139.825	4.39
A	48	51.2	51.0	51.1	53.0	53.0	54.9	6.458	11.74
Y	28	27.6	27.3	27.6	27.7	27.3	28.4	-1.786	-0.71
M	14	6.8	5.8	6.2	6.6	6.2	6.3	-55.238	-54.52
V	72	65.0	64.3	65.0	66.5	67.1	69.5	-10.046	-5.97
F	24	24.2	24.2	23.8	24.5	24.7	25.7	0.278	4.03
I	16	15.7	15.6	15.9	16.1	16.4	16.7	-1.667	2.50
L	54	56.0	55.5	56.1	58.0	58.5	60.6	3.457	9.32
K	54	56.2	57.3	40.1	60.7	65.5	42.0	6.033	5.64

5. Discussion

According to the global guidelines on quality considerations in demonstrating biosimilarity, the final dosage of the proposed product must be analyzed to follow that of the branded version and could affect the immunogenicity of the biodrugs. Previous studies have suggested that a decrease in the concentration of etanercept may decrease conformational stability (T_m) by increasing hydrodynamic size and zeta potential, and decreasing secondary structural stability (17,18). Therefore, equality

of the samples concentrations is of importance, which was stated to be in the range in this study.

Analysis of the product related impurities such as truncated forms, misfolded, aggregated and degraded etanercept was done by several orthogonal methods such as SDS-PAGE, CE-SDS and HIC/SE-HPLC. The relative percentages of product related HMWC and LMWC of reference and biosimilar products were analyzed using SDS-PAGE. In all the samples, in reducing SDS-PAGE, the major monomer band of 75 kDa was ≥ 88%, HMWC ≤ 5% and LMWC ≤ 9% of the total protein. Based on SDS-PAGE scanning densitometry results shown in **Table 1**, the six samples had insignificant variance in the content of HMWC and LMWC impurities. The data from capillary electrophoresis analysis indicated that the electrophoretic profiles of the reference and biosimilar proteins were similar (**Fig.2**), therefore the level of purity in Altebrel™ can be assumed similar to that of the Enbrel® samples.

Moreover, SE-HPLC and HIC-HPLC were performed for analyzing the size variants of the etanercept samples. SE-HPLC can separates the aggregated, etanercept monomer and the degraded forms from each other. Based on the SE-HPLC results (**Fig. 3** and **Table 2**), the relative AUC for aggregated, monomer and degraded forms of Altebrel™ and Enbrel® were comparable. According to etanercept monograph based on European Pharmacopoeia, the AUC of the aggregates must be lower than 8% of the total AUC or in the other word, the sum of monomer and degraded forms must be higher than 92% of the whole AUC of the peaks (19). HIC-HPLC can detect the misfolded, truncated and aggregated forms of etanercept, which are often due to the manufacturing conditions. These impurities are mainly produced in cell culture process and should be removed in the purification process.

As it was stated, aggregation, fragmentation, and proteolytic cleavage are regular problems observed in proteins, with potential direct or indirect influence on efficacy, safety and potency of the biological drugs. Regarding the cellular process involved in biopharmaceutical proteins production, heterogeneity in biodrugs is expected in the final drug dosage. Having lower levels of product/process-related impurities than the limitations is a critical parameter demanded by the regulatory agencies for proving the biosimilarity. As an effort to compare purity of the proposed biosimilar and reference products, orthogonal methods of SDS-PAGE, CE-SDS and SE/HIC-HPLC were employed in this study. According to the obtained results, Altebrel™ and Enbrel® exhibited similar patterns of product related variants and the purity of the final product was comparable and within the acceptable range for etanercept according to its specification.

Analysis of the etanercept affinity to TNFα was performed to compare the proposed biosimilar and the reference product kinetics of binding in a quantitative manner using SPR technology. SPR is a powerful method for quantifying the affinity of a ligand to its analyte. An appropriate curvature that starts from the lowest amounts of analyte to the highest amount that saturates the attached ligand is a gold parameter for tuning the SPR analysis. Moreover, a long dissociation phase is needed for the molecules which dissociate very slowly. Etanercept has a high affinity to TNFα in the range of picomolar that makes it a hard molecule for studying the affinity. The results presented in table 3 implied comparable KD values for Enbrel® and Altebrel™ samples. *In vitro* functional characterization of the biosimilar product is one of the key features required from the regulatory. Affinity analysis based on interactions between TNFα and etanercept products is employed to evaluate the binding properties of the drug in real time (20). As a bivalent receptor, etanercept forms a 1:1 complex with the TNF homotrimer, in which two of the three receptor binding sites on TNFα are occupied by etanercept, and the third receptor binding site is open (21). The high affinity between the two molecules makes it difficult to assess dissociation rate in a short period. Therefore, longer dissociation time was applied to provide enough time for etanercept to dissociate from immobilized TNF. Intrinsic binding affinities of Altebrel™ and Enbrel® were determined on a SPR instrument via kinetic titration and the results confirmed that Altebrel™ binds to TNFα with an affinity similar to the Enbrel®. In order to assessing the biological activities of Altebrel™ and Enbrel®, biological assays were performed in a side-by-side manner. Biological activity was evaluated based on measuring etanercept capability to neutralize the necroptotic effects of TNFα on the L929 cells. Dose response curve has been determined for each sample and compared by that of the reference standard in the four parameter logistic fit mode and the confidence limit of 95%. Specific biological activity (potency) of sample was calculated and reported in table 4. The results suggested that the potency of Altebrel™ is equivalent to that of the reference product. Based on the etanercept specification, the relative biological activity of biosimilar samples should be between 80% to 120% of the reference drug. Therefore, all the Altebrel™ samples have the potency within the range and could be considered similar to the reference.

Far-UV CD spectroscopy provides analytical data on the secondary structure of proteins. Production of biologics in living organisms entails an inherent degree of structural heterogeneity. The final product is a mixture of proteoforms with different PTM variants. A biosimilar must be proven to be equivalent to the reference product in terms of structural characteristics (22). Based on the results from far-UV CD, it was concluded that the secondary structure of Altebrel™ is similar to Enbrel®. For all tested samples of Altebrel™ and Enbrel®, the far-UV CD spectra indicated a folded protein with a high proportion of α-helical secondary structure. The high degree of congruence between far-UV CD spectra of Altebrel™ and Enbrel® confirmed their similarity with respect to their secondary structure (Table 5). Although widely used for the analysis of protein secondary structure, circular dichroism imparts certain limitations for large complex proteins such as etanercept. Thus, more sophisticated methods including hydrogen/deuterium exchange and differential scanning calorimetry in combination with mass spectrometry has been increasingly demanded by the regulatory agencies to perform a precise structural comparison between a biosimilar protein and the reference medicinal product (16).

Amino acid analysis is a perfect method for analyzing the amino acid contents of the peptides and proteins. It is a very powerful method for the peptides and small proteins but very hard to set up for the large ones like etanercept. By looking at the table 6, it can be inferred that the recognized amino acids could be separated to different groups. Group one contains the amino acids which were recognized well, with a compositional error of ≤ 15%, comparing to the known values from etanercept sequence. In this group, the amino acids N+D (asparagine is converted to aspartic acid during acid hydrolysis process), E+Q (glutamine is converted to glutamic acid during acid hydrolysis), S, H, G, T, A, Y, V, F, I, L and K can be mentioned. Methionine is the amino acid that was recognized in both samples with a high compositional error. This can be due to the susceptibility of methionine to oxidation that cause a change in hydrophobicity of the amino acid, therefore would change its position in the RP-HPLC. Arginine is an amino acid which was evaluated in the Enbrel® samples well but not in Altebrel™ samples. Since the differences is much high, it should be related to a modification that only occurred in Altebrel™ which results in the deviated evaluation of arginine.

As a prerequisite for determination of biosimilarity, protein primary structure was compared between Altebrel™ and Enbrel®. Considering the amino acid analysis limitation for the large proteins, it is a method for evaluation of amino acid content of a protein when enough data about the primary structure is not available. It can also be used for confirmation of the primary sequence when the primary sequence is known. The results of amino acid analysis using RP-HPLC corroborated with the theoretical sequence of etanercept, indicating similar primary structure and amino acid content for Altebrel™ and the reference product.

6. Conclusion

About a decade after approval of the first biosimilar by EMA, the debate on the type and extent of analytical, preclinical, and clinical details demanded for authorization of biosimilars still remains (10). However, the mutual regulatory outlook is emphasized on the identical structure, function and quality, comparable safety and equivalent efficacy to an already licensed reference medicine (7). Herein, Altebrel™ as a proposed etanercept biosimilar was characterized in a head-to-head comparability studies. Overall, our data provided non-clinical analytical evidence for biosimilarity of Altebrel™ to the reference biological product (Enbrel®) in terms of purity, structure and potency. In terms of fragmentation, four orthogonal methods of SDS-PAGE, CE-SDS, SE/HIC-HPLC showed equivalent patterns of size variants for both molecules. Application of amino acid analysis method and CD spectroscopy proved that the primary and secondary structure of Altebrel™ and Enbrel® are identical respectively. Furthermore, the two molecules showed comparable biological potency, evaluated by SPR and *in vitro* bioassay. It is noteworthy to mention that besides the applied methods, more data on the amino acid sequence and the PTMs of etanercept beside the clinical and non-clinical studies are needed. The data about the analysis of etanercept primary sequence, glycosylation and other PTM are under publication, which showed the similarity of Altebrel™ and Enbrel®. This study represents the evaluations on the several quality attributes of etanercept in which the proposed biosimilar and the reference product were comparable. Therefore, data from a panel of analytical methods and *in-vitro* functional assays concluded that the applied analysis states a high similarity of Altebrel™ to Enbrel®.

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